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Coordinate Regulation of *Salmonella enterica* Serovar Typhimurium Invasion of Epithelial Cells by the Arp2/3 Complex and Rho GTPases

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***Salmonella enterica* serovar Typhimurium can infect epithelial cells via the basolateral surface after breaching the intestinal epithelium, yet little is known about this process. Here, we show that actin polymerization driven by the Arp2/3 complex is critical to both basolateral and apical bacterial invasion of polarized MDCK cells. While there is also a dependence upon toxin B-sensitive Rho GTPases, none of the four GTPases known to be activated by *S. enterica* serovar Typhimurium SopE are individually required for basolateral internalization. These results underscore that the specific factors required for *Salmonella* invasion differ between membrane domains of polarized epithelia.**

Nontyphoidal strains of *Salmonella* such as *Salmonella enterica* serovar Typhimurium are a common cause of food-borne gastroenteritis, with the number of human cases per year in the United States estimated at 1 to 3 million (23). Ingested salmonellae bind to the surface of epithelial cells of the small intestine and translocate a set of bacterial proteins through the type III secretory apparatus encoded by the *Salmonella* pathogenicity island-1 (SPI-1) chromosomal locus (8, 12). These proteins directly modulate host cell actin dynamics, leading to extensive membrane ruffling at the site of attachment of the bacteria and subsequent bacterial engulfment (15, 19, 21, 40, 47, 48).

While salmonellae primarily interact with the lumenal (apical) surface of the intestinal epithelium, in vivo they also gain access to the basolateral surfaces of enterocytes. This can be accomplished directly, by bacterial transport through microfold (M) cells of Peyer's patches in the small intestine (4, 7, 24, 45). M cells are specialized for engulfing foreign objects in the lumen and transporting them to subepithelial lymphocytes; it is thought that transport through M cells provides an important mechanism by which enteric pathogens like *Shigella* and *Yersinia* establish systemic infection (36, 39). Indirect access to the basolateral surfaces of enterocytes may also occur as a consequence of M cell destruction or the migration of neutrophils through the epithelium, both of which compromise the barrier function of the intestinal monolayer (5, 24, 31).

Our laboratory has begun to characterize the cellular proteins coopted by invasive salmonellae to effect their internalization by epithelial cells. Using Madin-Darby canine kidney (MDCK) cells as a model for polarized epithelia, we have shown that bacterial internalization at the apical domain requires the Rho GTPase Rac1, while neither Rac1 nor Cdc42 activity is solely required for invasion at the basolateral pole (10). In the present study, we sought to characterize cellular proteins involved in basolateral internalization of *Salmonella* and have established a role for the Arp2/3 complex, which mediates the formation of branched actin meshworks, in this process.

First, we visualized basolateral bacterial internalization in our in vitro polarized cell system. MDCK cells expressing the tetracycline-regulated transactivator (T23 MDCK) were grown to confluency on collagen-coated filter supports with 3-μm-diameter pores (Costar, Cambridge, Mass.), which are large enough to accommodate bacterial movement through the filter, as previously described (10). In all experiments, MDCK monolayers became completely polarized by 4 days after plating, as evidenced by transepithelial resistance levels of greater than 200 $\Omega \cdot cm^2$. Cells were infected basolaterally with *S. enterica* serovar Typhimurium strain SL1344, grown under microaerophilic conditions (as described in reference 26), at a multiplicity of infection of 100 for 30 min at 37°C and then fixed and processed for electron or confocal immunofluorescence microscopy as described previously (10). Electron microscopy revealed that basolateral *Salmonella* induced the formation of membrane protrusions that extended into the filter to engulf invading bacteria (Fig. 1A). Similar to the protrusions observed during apical infection, these basolateral protrusions were dependent on rearrangements of the actin cytoskeleton, as detected using fluorescein-coupled phalloidin (Molecular Probes, Eugene, Oreg.); bacteria were recognized with an anti-*Salmonella* polyclonal antiserum (Difco, Detroit, Mich.) followed by Texas Red-coupled anti-rabbit immunoglobulin G (IgG) (Jackson Immunoresearch, West Grove, Pa.) (Fig. 1B). These actin-based structures appeared less extensive than those previously seen at the apical aspect of polarized cells, probably as a consequence of physical constraints imposed by the filter and neighboring cells (10) (compare the apical and basolateral invasion structures in Fig. 2A). We anticipate that salmonellae which gain access to the basal surface of the intestinal epithelium in vivo induce similar structures, although this has not been fully examined.

Previous reports using in vitro models indicate that *Salmo-*

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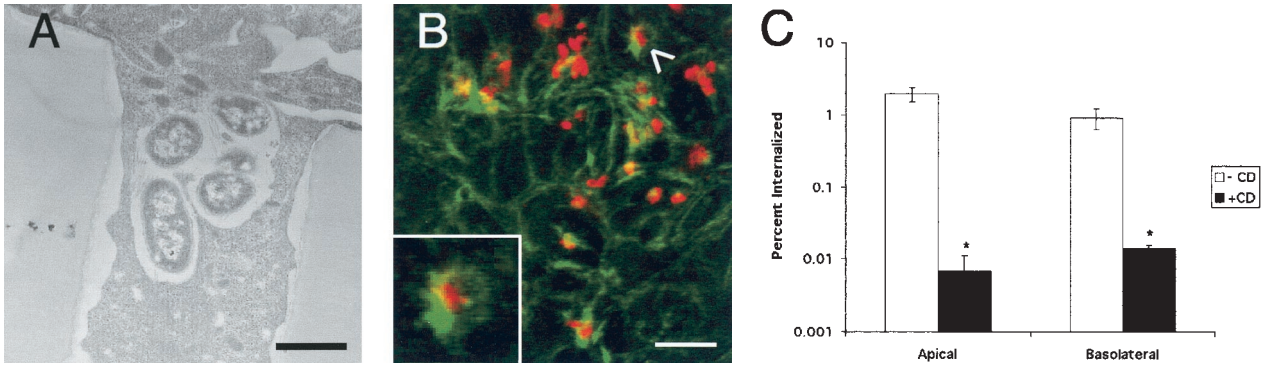

FIG. 1. *S. enterica* serovar Typhimurium induces the formation of actin-dependent membrane protrusions during basolateral invasion of polarized MDCK cells. (A and B) MDCK cells seeded on 3-μm-pore-size filter supports were infected from the basolateral pole with *Salmonella* and then fixed and processed for transmission electron microscopy (A) or confocal fluorescence microscopy (B). The red areas in panel B represent bacteria, and the green areas represent filamentous actin. The arrowhead in panel B indicates the region magnified in the inset. Bars: 1 μm (A), 10 μm (B). (C) MDCK cells were treated with 10 μg of cytochalasin D (CD)/ml and then subjected to a gentamicin resistance invasion assay. White bars, untreated cells; black bars, cells treated with CD. Results are expressed as mean invasion percentages ± standard deviations. *, $P < 0.025$ (treated cells versus untreated control cells; Student's *t* test).

nella invasion at the basal aspect of polarized epithelial cells occurs with the same efficiency as at the apical aspect (17). In correlation, we found that both apical and basolateral *Salmonella* internalization required de novo actin polymerization. MDCK cells cultured to confluency on filter supports were treated with the actin-depolymerizing agent cytochalasin D (Sigma Chemical Co., St. Louis, Mo.) at 10 μg/ml for 1 h prior to apical or basolateral infection with *Salmonella*, and the number of internalized bacteria was quantified using the gentamicin resistance assay as described previously (10). As seen in Fig. 1C, treatment with cytochalasin D inhibited both apical and basolateral bacterial internalization significantly, to less than 1% of the control, untreated levels ($P < 0.025$ versus control for each condition; Student's *t* test). Therefore, salmonellae may utilize the same tools to enter at the basal pole of polarized epithelial cells as they do at the apical pole or at the surface of nonpolarized cells.

The requirement for actin polymerization during *Salmonella* invasion of polarized epithelial cells prompted us to examine the Arp2/3 complex in this process. This seven-subunit complex facilitates the rapid growth of actin filaments during dynamic cellular events such as phagocytosis and chemotaxis by nucleating actin filament growth from the sides of existing filaments, thereby forming a dendritic actin network (22, 41, 42). Activation of the Arp2/3 complex requires accessory proteins, primarily those of the WASp and Scar/WAVE family, which serve both to target the complex to the correct subcellular destination and to stimulate its activity (44). First, we examined the localization of the Arp2/3 complex in *Salmonella*-infected MDCK cells by confocal fluorescence microscopy. The Arp2/3 complex was recognized using polyclonal antiserum to the p21 subunit followed by Texas Red-coupled anti-rabbit IgG; bacteria were detected using a monoclonal IgA anti-*Salmonella* antibody followed by fluorescein-coupled anti-mouse IgA (Southern Biotechnology, Birmingham, Ala.). As seen in Fig. 2A, the Arp2/3 complex was recruited to *Salmonella*-induced ruffles in response to both apically and basolaterally invasive bacteria. These findings are in agreement with those of Stender et al. (40), who showed that an antibody to a different subunit of the complex (p41) localizes to ruffles in nonpolarized cells infected with *Salmonella*, and suggest that the Arp2/3 complex is positioned to play a key role in the actin reorganization required for bacterial internalization.

To examine this possibility, *Salmonella* invasion was measured in polarized MDCK cells expressing domains of the Scar1 protein. Two domains of WASp/Scar proteins are required for Arp2/3 complex activation: the W domain, which binds G-actin, and the adjacent A domain, which binds the Arp2/3 complex. When ectopically expressed, a C-terminal fragment of Scar1 containing both the W and A domains (WA) sequesters the Arp2/3 complex from endogenous WASp/SCAR proteins, thereby stimulating unregulated and delocalized actin polymerization (Fig. 2B) (27, 28, 30). The W domain alone and the tandem WA domains of Scar1 were subcloned from pRK5 into the adenoviral shuttle vector pAdTet to include an N-terminal myc epitope tag (*Cla*I-*Hin*dIII restriction digest). These constructs were then used to generate recombinant tetracycline-regulated adenoviruses as previously described (20). T23 MDCK cells were infected with the appropriate adenoviruses in the presence or absence of 20 ng of doxycycline/ml to repress or induce transgene expression, respectively (11). Overexpression of Scar WA inhibited *Salmonella* internalization at both apical and basolateral membrane domains by 80% compared to that of cells overexpressing Scar W, which is not inhibitory for the complex ($P < 0.05$ for Scar WA with doxycycline versus without doxycycline for each condition; Student's *t* test) (Fig. 2B). Expression levels of the adenovirally expressed constructs were equivalent, as assessed by immunoblotting (Fig. 2B, upper right). These results provide evidence that the actin polymerization necessary for *Salmonella* internalization into polarized cells requires the Arp2/3 complex.

In many circumstances, Arp2/3 complex-mediated actin remodeling occurs downstream of signaling by members of the Rho family of monomeric GTPases, particularly Rac1 and Cdc42 (34, 37, 44). When in their GTP-bound, active state, these proteins facilitate the unfolding of WASp/SCAR proteins, leading to exposure of their WA domains. Work from

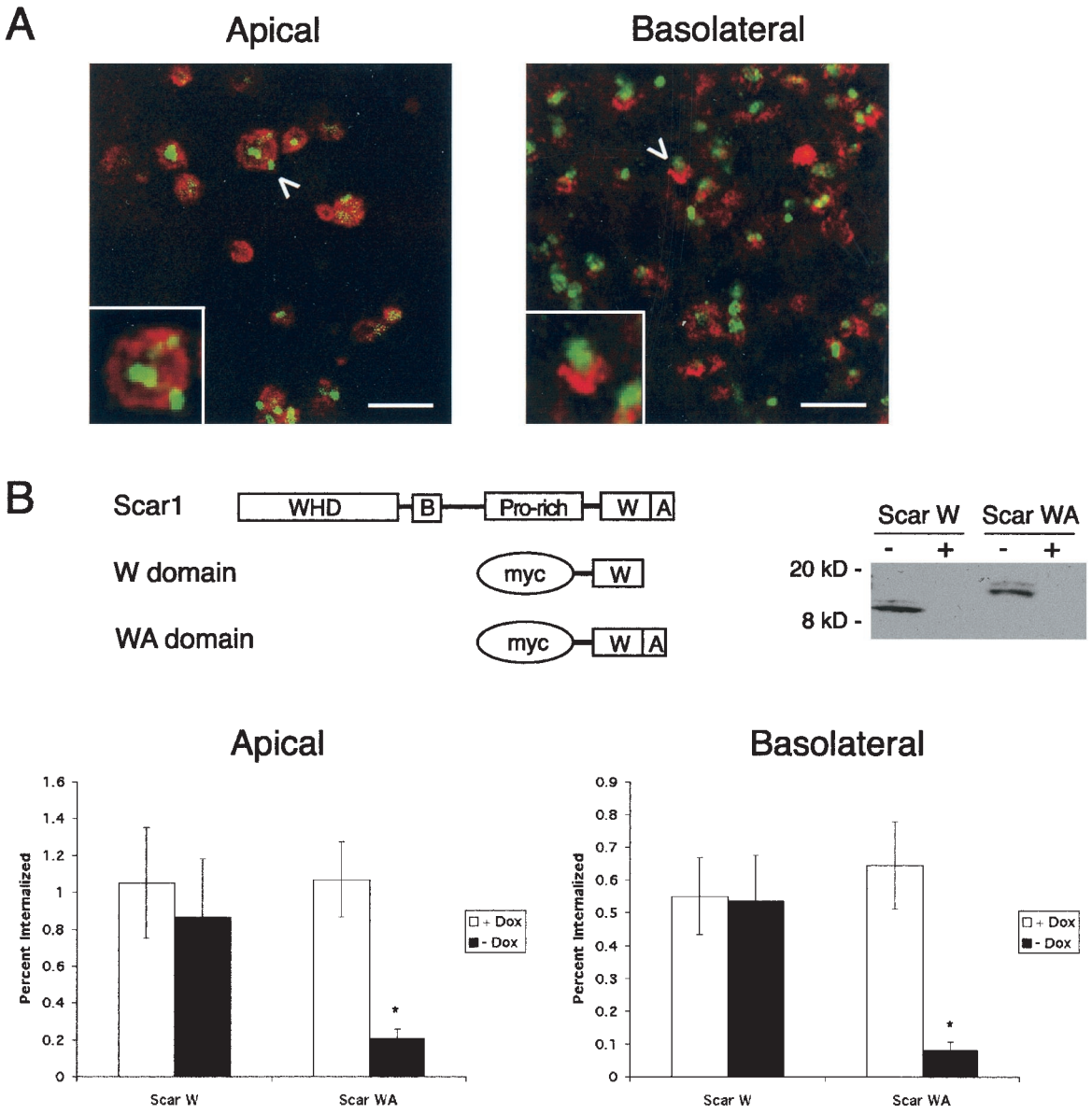

FIG. 2. The Arp2/3 complex is required for *Salmonella* invasion of polarized epithelial cells. (A) MDCK cells were infected at the apical (left panel) or basolateral (right panel) pole with *Salmonella* as described for Fig. 1B. The red areas in panels A and B represent the p21 subunit of the Arp2/3 complex; the green areas represent bacteria. Bars, 10 μm. Arrowheads indicate regions magnified in insets. (B) Top left panel: schematic of full-length Scar1 and adenovirally expressed Scar1 constructs. WHD, WAVE/Scar homology domain; B, basic domain; Pro-rich, proline-rich (SH3-binding) domain; W, G-actin-binding W domain; A, Arp2/3 complex-binding acidic domain. Top right panel: lysates from MDCK cells infected with recombinant adenoviruses encoding the W or WA domains from Scar1 were separated on a sodium dodecyl sulfate–4 to 20% polyacrylamide gradient gel and immunoblotted with an anti-myc (9E10) antibody. −, no doxycycline (to induce transgene expression); +, maintenance in doxycycline (to repress expression). Bottom panel: MDCK cells infected with Scar W or Scar WA adenoviruses were subjected to a gentamicin resistance invasion assay as described for Fig. 1C. White bars, cells were maintained in 20 ng of doxycycline/ml to repress transgene expression; black bars, no doxycycline. *, $P < 0.05$ compared to control in the presence of doxycycline.

many laboratories has revealed that *Salmonella* internalization requires Rho GTPase activity, but the specific family members involved vary among cell types. For instance, invasion of nonpolarized tissue culture cell lines can be inhibited by dominant-negative mutants of both Rac1 and Cdc42 (6). In contrast, we recently showed that apical internalization of *Salmonella* into polarized MDCK monolayers requires Rac1 but not Cdc42 (10). In that same study, we found that basolateral *Salmonella* does not require the activity of either Rac1 or Cdc42 to gain access at this membrane domain, raising the possibility that basolateral invasion could, uniquely, be independent of Rho GTPase activity. However, we observed that the *S. enterica* serovar Typhimurium SPI-1 secreted effector protein SopE participates in both apical and basolateral bacterial internalization; SopE and the closely related SopE2 activate Rho GTPases directly by promoting GTP binding (14, 19, 40). This

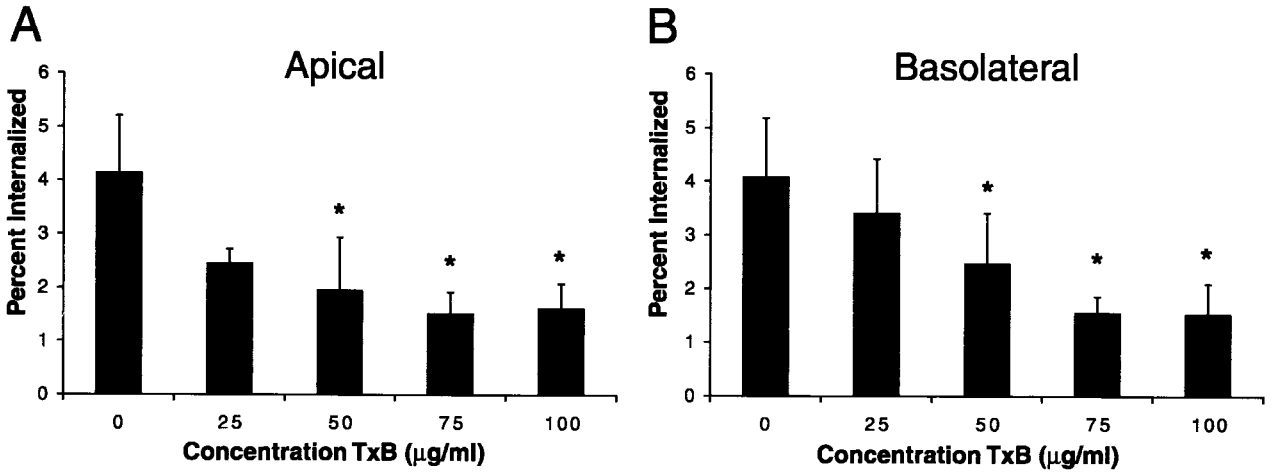

FIG. 3. Treatment of MDCK cells with *C. difficile* toxin B inhibits basolateral internalization of *Salmonella*. MDCK cells were treated with toxin B at the indicated concentrations and then subjected to a gentamicin resistance invasion assay as described for Fig. 1C. Cells were infected apically (A) or basolaterally (B). *, $P < 0.05$ versus untreated control.

suggested that a SopE-dependent GTPase (or GTPases) is involved in basolateral *Salmonella* invasion.

To directly address this question, bacterial invasion into polarized MDCK monolayers was measured using the gentamicin resistance assay, following a 3-hour treatment with toxin B from *Clostridium difficile* (List Biological, Campbell, Calif.). This clostridial toxin monoglucosylates Rho family members at a conserved threonine residue in the GTPase nucleotide binding pocket, rendering them inactive (2). As expected given the involvement of Rac1 in apical *Salmonella* internalization, toxin B treatment inhibited apical bacterial entry in a dose-dependent manner ($P < 0.05$ for treated conditions versus untreated control conditions; Student's *t* test) (Fig. 3A). Notably, basolateral bacterial entry into MDCK cells was also inhibited with increasing concentrations of toxin, reaching maximum inhibition at ~75 μg/ml (Fig. 3B). Values of 50% inhibitory concentrations for both apical and basolateral invasion were calculated at approximately 60 μg/ml, similar to the dosages needed to inhibit entry of other bacterial pathogens such as *Pseudomonas aeruginosa* into polarized MDCK monolayers (25). Toxin-mediated inhibition of Rho GTPases was complete at 75 μg/ml, since no further decrease in invasion frequency was observed at higher concentrations. At this concentration of toxin B, *Salmonella* invasion was reduced to 20% of control (untreated) levels, which correlates well with the invasion deficiency of a *sopE sopE2* mutant strain of *S. enterica* serovar Typhimurium (40). Toxin B-mediated inhibition of *Salmonella* internalization was not as great as that seen with cytochalasin D (Fig. 1), since SPI-1 effector proteins in addition to SopE/E2 are required for maximal bacterial engulfment by nonphagocytic cells (47). These results indicate that SopE-dependent, toxin B-sensitive Rho family GTPases contribute equally to apical and basolateral *Salmonella* internalization by polarized epithelial cells.

Hardt et al. reported that *S. enterica* serovar Typhimurium SopE has in vitro nucleotide exchange activity on four Rho family members: Rac1, Cdc42, RhoA, and RhoG (19). Having examined Rac1 and Cdc42 in our previous study, we analyzed the contribution of RhoA and RhoG to basolateral *Salmonella* invasion. RhoA is necessary for the formation of stress fibers and focal adhesions in fibroblasts (18). Epithelial internalization of *Shigella flexneri*, which induces membrane ruffles structurally similar to those evoked by *Salmonella*, requires RhoA activity; notably, *Shigella* invades epithelia exclusively at the basal cell surface (1, 35, 46). Using a procedure described earlier for precipitation of Rac1 (10, 38), we measured the nucleotide binding state of RhoA from infected MDCK cell lysates by precipitating the endogenous, activated (GTP-bound) protein with the RhoA binding domain of rhotekin. Changes in the activation state of RhoA were expressed as a ratio of GTP-bound RhoA to total RhoA in a fraction of the cell lysate. Both apical and basolateral *Salmonella* infection for 30 min promoted a modest (1.4-fold) increase in activated RhoA in cell lysates compared to that seen with control (uninfected) cells. However, this change was not significant compared to that seen with cells infected with a bacterial strain from which the SPI-1 regulator *hilA* was deleted; this strain is unable to produce invasion-promoting effector proteins, including *sopE* (10) (Fig. 4A). No change in the activation state of RhoA was observed even at times of invasion when Rac1 and/or Cdc42 becomes activated (10). Furthermore, overexpression of a dominant-negative RhoA construct (N19) did not inhibit bacterial invasion at either the apical or basolateral cell surface (data not shown). Therefore, although SopE has the ability to activate RhoA in vitro, the GTPase does not become activated in vivo during bacterial invasion of polarized epithelial cells.

In fibroblasts, RhoG activation stimulates the formation of Rac1-dependent membrane ruffles and Cdc42-dependent filopodia (16). To examine the contribution of RhoG to *Salmonella* invasion, we developed clonal MDCK cell lines inducibly expressing the RhoG interacting protein RhoG IP122. Overexpression of the RhoG binding domain of this protein has been shown by Blangy et al. to prevent formation of actin cytoskeletal rearrangements downstream of activation of the GTPase (3). We felt that use of the RhoG IP122 construct was

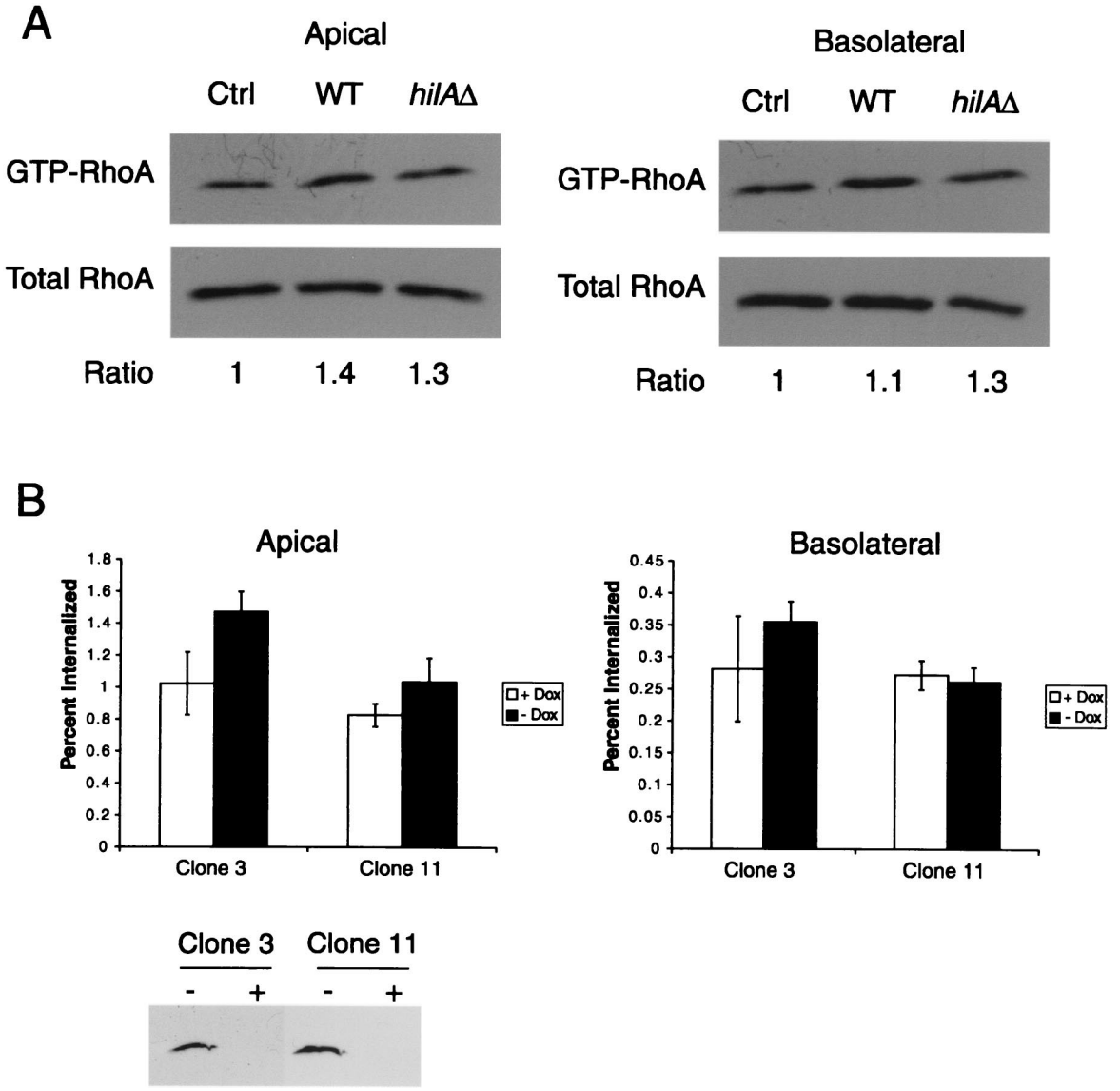

FIG. 4. Neither RhoA nor RhoG is required for basolateral *Salmonella* invasion of polarized MDCK cells. (A) MDCK cells were infected with *Salmonella* at the apical or basolateral pole and then lysed and subjected to a RhoA-GTP precipitation assay using the N terminus of rhotekin. Upper panels, GTP-RhoA; bottom panels, total RhoA in lysate; Ratio, GTP-RhoA/total RhoA; Ctrl, uninfected cells; WT, wild-type *S. enterica* serovar Typhimurium strain SL1344; *hilA*Δ, *S. enterica* serovar Typhimurium strain VV341 lacking functional *hilA*. (B) Top panel, MDCK cells inducibly expressing the RhoG interacting protein RhoG IP122 were isolated and subjected to a gentamicin resistance invasion assay as described for Fig. 1C. Results from two representative clones are shown. Bottom, lysates from clones 3 and 11 were separated on a SDS–10% polyacrylamide gel and then immunoblotted with an anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) to recognize the tagged construct. −, no doxycycline (to induce transgene expression); +, maintenance in doxycycline (to repress expression).

preferable to overexpression of a dominant-negative RhoG mutant, since the latter could nonspecifically sequester the SopE/E2 exchange factors during invasion. Four RhoG IP122-expressing MDCK clones were isolated and subjected to gentamicin resistance invasion assays with *Salmonella*. These clones expressed similar amounts of protein, as detected by immunoblotting (Fig. 4B). The results for two representative clones (clones 3 and 11) are shown in Fig. 4B; these results reveal that RhoG activity is not required for apical or basolateral invasion of polarized cells. These findings strongly suggest that *Salmonella* invasion at the basolateral pole does not depend upon the activity of RhoG or RhoA even though it does involve toxin B-sensitive Rho GTPase activity.

These results provide two important conclusions regarding *Salmonella* invasion of polarized epithelia. First, bacterial internalization at both domains of polarized cells requires activation of the Arp2/3 complex. Second, basolateral internalization of *Salmonella* utilizes toxin B-sensitive Rho GTPases but does not individually require Rac1, Cdc42, RhoA, or RhoG. We envision two possibilities for what might be occurring at the basolateral surface during *Salmonella* infection. In one scenario, a toxin B-sensitive Rho family member that has not

yet been examined is necessary for basolateral bacterial internalization. This GTPase would be a substrate for SopE and would presumably stimulate actin polymerization by activating WASp and/or Scar/WAVE family members. Although we have now examined the four Rho GTPases shown by Hardt et al. (19) to serve as substrates for SopE, over fifteen Rho family members have been identified to date, some of which might be found to have essential roles in basolateral actin remodeling. An alternative hypothesis is that the activities of multiple Rho GTPases are simultaneously coordinated during basolateral invasion. We found previously that Rac1 and Cdc42 are both activated during basolateral bacterial internalization but that neither GTPase alone was important for entry (10). However, many Rho GTPases act together to promote important cellular processes, including receptor-mediated phagocytosis (9, 29). The development of new tools to specifically inactivate or sequester subsets of Rho GTPases will certainly contribute to addressing this point. These two hypotheses are not mutually exclusive, and we anticipate that as more becomes known about Rho GTPases, we will be able to identify the toxin B-sensitive family members participating in basolateral *Salmonella* invasion.

As mentioned above, the Arp2/3 complex is activated by members of the WASp and Scar/WAVE family of proteins. While they share significant structural similarity, especially at their C termini, these proteins are activated in response to different GTPase-dependent signals. For instance, WASp and N-WASp activation is dependent on Cdc42 binding to a conserved domain in the middle of the protein (32, 37, 43). In contrast, Scar/WAVE proteins form a complex with Rac1 via adaptor proteins such as IRSp53 or Nck (13, 33, 34). Once the appropriate antibodies have been developed, we intend to identify whether this latter complex is responsible for apical internalization of *Salmonella* in polarized epithelial cells. It will also be illuminating to determine which WASp/SCAR family member localizes to protrusions induced by basolaterally invasive bacteria, which may shed light on other regulators of this event. In addition to enhancing our knowledge of the invasion mechanisms of an important bacterial pathogen, we anticipate that these findings will also provide insight into how a polarized actin cytoskeleton is established and maintained in mature epithelial tissues.

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